



Europäisches Patentamt  
European Patent Office  
Office européen des brevets



Publication number: **0 170 917 B1**

(12)

# EUROPEAN PATENT SPECIFICATION

- (45) Date of publication of patent specification: 29.05.91 (51) Int. Cl.<sup>5</sup>: **C07K 15/26, C12N 15/00, C12P 21/02, C12N 1/20, //C12R1:19**
- (21) Application number: **85108541.5**
- (22) Date of filing: **09.07.85**

The file contains technical information submitted after the application was filed and not included in this specification

(54) Novel human interferon-gamma polypeptide derivative.

- (30) Priority: 11.07.84 JP 143950/84
- (43) Date of publication of application: 12.02.86 Bulletin 86/07
- (45) Publication of the grant of the patent: 29.05.91 Bulletin 91/22
- (84) Designated Contracting States: AT BE CH DE FR GB IT LI NL SE
- (56) References cited:  
EP-A- 0 083 777  
EP-A- 0 095 350  
EP-A- 0 103 898  
EP-A- 0 112 976  
EP-A- 0 146 944

- (73) Proprietor: KYOWA HAKKO KOGYO CO., LTD.  
6-1, Ohte-Machi Itchome  
Chiyoda-ku Tokyo(JP)
- (72) Inventor: Itoh, Seiga  
218-14, Aza Hachimannishi Aihara  
Sagamihara-shi Kanagawa-ken(JP)  
Inventor: Takeichi, Yasutoshi  
4-17-9, Morino  
Machida-shi Tokyo(JP)  
Inventor: Sato, Moriyuki  
2730-15, Naruse  
Machida-shi Tokyo(JP)
- (74) Representative: Vossius & Partner  
Siebertstrasse 4 P.O. Box 86 07 67  
W-8000 München 86(DE)

EP 0 170 917 B1

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

Rank Xerox (UK) Business Services

BEST AVAILABLE COPY

## Description

Interferons (referred to as IFN hereinafter) so far known can be classified into three groups, i.e. IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$ . IFN- $\alpha$  is mainly produced from leukocytes, IFN- $\beta$  from fibroblasts and IFN- $\gamma$  from T-lymphocytes. These IFNs have been noted as biologically active substances having anti-virus activity, activating activities upon natural killer cells and macrophages, antitumor activity, and the like.

As for IFN- $\gamma$ , there has been a report that it has a stronger cell-inhibiting activity than other IFNs based on the experiment using animal cells [B.Y. Rubin and S.L. Gupta: Proc. Natl. Acad. Sci., USA, 77, 5928 - 5932 (1980)]. Furthermore, cloning of an IFN- $\gamma$  cDNA and determination of its base sequence were recently reported [P.W. Gray, et al.: Nature 295, 503 (1982), R. Devos, et al.: Nucleic Acids Research 10, 2467 (1982)].

The present inventors have independently cloned a cDNA coding for a novel IFN- $\gamma$  wherein, as apparent from the base sequence illustrated in Table 1, the ninth amino acid of the mature IFN- $\gamma$  reported by Devos, et al., Lysine (Lys) (AAA), is replaced with glutamine (Gln) (CAA). Further, the IFN- $\gamma$  cDNA was incorporated into vector pKYP-10 having a tryptophan promoter (Japanese Published Unexamined Patent Application No. 110600/83) and mass production of the IFN- $\gamma$  in *Escherichia coli* has been achieved.

Thereafter, the present inventors have studied the production of derivatives of IFN- $\gamma$  polypeptide using the IFN- $\gamma$  cDNA illustrated in Table 1 as a starting material.

It was reported that deletion of 11 amino acids from the C-terminal of IFN- $\alpha$  decreased specific activity to one-third [A.E. Franke, et al.: DNA 1, 223 - 230 (1982)], whereas addition of 18 amino acids to the N-terminal of IFN- $\alpha$  did not change specific activity [R.M. King, et al.: J. Gen. Virol. 64, 1815 - 1818 (1983)].

The present inventors have found that deletion of 5 amino acids from the N-terminal of IFN- $\beta$  decreased specific activity to about 1/100 [T. Nishi, et al.: DNA 2, 265 - 273 (1983)] and addition of 7 amino acids to the N-terminal of IFN- $\beta$  decreased specific activity to about 1/10 [S. Itoh, et al.: DNA 3, 157 - 165 (1984)].

EP-A2 95 350 discloses a human IFN- $\gamma$ -like polypeptide. The amino acid sequence thereof is given on page 2 of EP-A2 95 350. The amino acid residue at position 9 is a Lys-residue (the derivatives of the present invention have a Gln-residue at position 9).

EP-A2 146 944, a document under Art. 54(3) EPC, discloses five derivatives of the human IFN- $\gamma$  polypeptide. All of them have a carboxy-terminus which is identical to the naturally occurring human IFN- $\gamma$ . Therefore, these derivatives differ from those of the present invention which display a deletion of the amino acid positions 138-146.

The present inventors have constructed a derivative of IFN- $\gamma$  wherein the third amino acid of IFN- $\gamma$  illustrated in Table 1, cysteine (Cys), was replaced by tyrosine (Tyr) (referred to as 3-Tyr-IFN- $\gamma$  hereinafter) and found that the specific activity was 2 - 4 times as strong as that of the parent IFN- $\gamma$ . Further, the derivatives wherein the Cys at the position 1 was replaced by serine (Ser) (1-Ser-IFN- $\gamma$ ), the Cys at the position 3 was replaced by Ser (3-Ser-IFN- $\gamma$ ), the Cys at the positions 1 and 3 were replaced by Ser (1,3-Ser-IFN- $\gamma$ ) and N-terminal amino acids of IFN- $\gamma$  illustrated in Table 1 were deleted were constructed. Equivalent or higher interferon activity was detected for all the derivatives compared with the starting IFN- $\gamma$ .

It has been known that murine mature IFN- $\gamma$  consists of 136 amino acids, which number is less by 10 than that of amino acids of human IFN- $\gamma$ , and is more heat-stable than human IFN- $\gamma$ . Murine IFN- $\gamma$  has Cys at the position 136 as well as at the positions 1 and 3 and these Cys form disulfide bonds in the molecule, which are considered to contribute to stabilization of IFN [D. Goeddel, et al.: Proc. Natl. Acad. Sci. USA 80, 5842 - 5846 (1984)].

The present inventors have prepared the molecular model of IFN- $\gamma$  and assumed that the formation of disulfide bond in the molecule of human IFN- $\gamma$  was possible by introducing Cys between the positions 135 and 138 from the N-terminus, preferably at the position 137. Several derivatives of IFN- $\gamma$  wherein C-terminal amino acids were deleted and Met at the position 137 was replaced by Cys have been found to have increased heat stability and specific activity.

The present invention relates to novel derivatives of the human IFN- $\gamma$  polypeptide which are encoded by the recombinant plasmid pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) or pGNB4 (TERM BP-549). The novel derivatives have high IFN- $\gamma$  activity and are expected to be useful as drugs such as anti-viral and anti-tumor agents.

Fig. 1 is a flow sheet for the construction of plasmid pGVT137. As the sites for *Hinf*I and *Taq*I, only those which are used for the construction are indicated.

Fig. 2 is a flow sheet for the construction of plasmid pGNC5. As the sites for *Taq*I, only those which are used for the construction are indicated.

Fig. 3 is a flow sheet for the construction of plasmid pGNB4. As the sites for *Taq*I, only those which are used for the construction are indicated.

Fig. 4 is a flow sheet for the construction of plasmid pGNA3. As the sites for *Taq*I, only those which are used for the construction are indicated.

An object of the present invention is to provide a recombinant plasmid wherein a DNA coding for a novel derivative of human IFN- $\gamma$  is incorporated, a microorganism containing the plasmid, a process for producing a novel derivative of human IFN- $\gamma$  polypeptide using the microorganism and the derivative of human IFN- $\gamma$  polypeptide per se.

Construction of the recombinant plasmid is carried out using cDNA obtained from messenger RNA coding for IFN- $\gamma$  by recombinant DNA technology or chromosomal DNA coding for IFN- $\gamma$  as a starting material.

In the present invention, any human IFN- $\gamma$  cDNA is employable and pIFN- $\gamma$ -G4 is preferably used. Escherichia coli containing pIFN- $\gamma$ -G4 has been deposited with the American Type Culture Collection, USA under accession number ATCC 39123.

The sequence of the IFN- $\gamma$  DNA in pIFN- $\gamma$ G4 was determined by the method of Maxam and Gilbert [Proc. Natl. Acad. Sci. 74, 560 (1977)] and is illustrated in Table 1.

Table 1

20 CACATTGTTCTGATCATCTGAAGATCAGCTATTAGAAGAGAAAGATCAGTTAAGTCCCTTTGGACCTGATCAGCTTGATACAAGAACTACTGATT  
 -20  
 met lys tyr thr ser tyr ile leu ala phe gln leu cys ile val leu  
 25 CAACTTCTTTGGCTTAATTCTCTCGGAAACG ATG AAA TAT ACA AGT TAT ATC TTG GCT TTT CAG CTC TGC ATC GTT TTG  
 1 10 20  
 gly ser leu gly cys tyr cys gln asp pro tyr val glu glu ala glu asn leu lys lys tyr phe asn ala  
 ggt tct ctt ggc tgt tac tgc cag gac cca tat gta caa gaa gca gaa aac ctt aag aaa tat ttt aat gca  
 1  
 30  
 gly his ser asp val ala asp asn gly thr leu phe leu gly ile leu lys asn trp lys glu glu ser asp  
 ggt cat tca gat gta gcg gat aat gga act ctt ttc tta ggc att ttg aag aat tgg aaa gag gag agt gac  
 50 60  
 arg lys ile met gln ser gln ile val ser phe tyr phe lys leu phe lys asn phe lys asp asp gln ser  
 aga aaa ata atg cag agc caa att gtc tcc ttt tac ttc aaa ctt ttt aaa aac ttt aaa gat gac cag agc  
 70 80 90  
 35 ile gln lys ser val glu thr ile lys glu asp met asn val lys phe phe asn ser asn lys lys lys arg  
 atc caa aag agt gtg gag acc atc aag gaa gac atg aat gtc aag ttt ttc aat agc aac aaa aag aaa cga  
 100 110  
 asp asp phe glu lys leu thr asn tyr ser val thr asp leu asn val gln arg lys ala ile his glu leu  
 gat gac ttc gaa aag ctg act aat tat tgc gta act gac ttg aat gtc caa cgc aaa gca ata cat gaa ctc  
 120 130 140  
 40 ile gln val met ala glu leu ser pro ala ala lys thr gly lys arg lys arg ser gln met leu phe arg  
 atc caa gtg atg gct gaa ctg tgc cca gca gct aaa aca ggg aag cga aaa agg agt cag atg ctg ttt cga  
 (7)  
 146  
 gly arg arg ala ser gln  
 45 ggt cga aga gca tcc cag taa tggttgtcctgcctgcaatatTTTGAATTTTAAATCTAAATCTATTATTAAATATTTAACATTATTTA  
 TATGGGGAATATATTTTAGACTCATCAATCAAATAAGTATTTATAATAGCAACTTTTGTGTAATGAAAATGAATATCTATTAATATATGTATTA  
 50 TTTATAATTCCTATATCTGTGACTGTCTCACTTAATCCTTTGTTTCTGACTAATTAGGCAAGGCTATGTGATTACAAGGCTTTATCTCAGGGG  
 CCAACTAGGCAGCCAACTAAGCAAGATCCCATGGGTTGTGTGTTTATTTCACCTTGATGATACAATGAACACTTATAAGTGAAGTGATACTATCC

5

78

15

28

25



40

45

51

54

Purification of the DNA fragments formed by digestion with restriction enzymes is carried out by low-

gelling-temperature agarose gel electrophoresis [L. Wieslander: Analytical Biochemistry 98, 305 (1979), hereinafter referred to as LGT method] or polyacrylamide gel electrophoresis.

Ligation of the DNA fragments is carried out with 0.3 - 10 units of T4 DNA ligase in a mixture of 2 - 200 mM, preferably 10 - 40 mM Tris-HCl (pH 6.1 - 9.5, preferably 7.0 - 8.0), 2 - 20 mM, preferably 5 - 10 mM MgCl<sub>2</sub>, 0.1 - 10 mM, preferably 0.5 - 2.0 mM ATP and 1 - 50 mM, preferably 5 - 10 mM dithiothreitol at 1 - 37 °C, preferably 3 - 20 °C for 15 minutes to 72 hours, preferably, 2 - 20 hours. The recombinant plasmid DNA formed by the ligation reaction is introduced into *Escherichia coli* by the transformation method of Cohen, et al. [S.N. Cohen, et al.: Proc. Natl. Acad. Sci. USA 69, 2110 (1972)], if necessary. Isolation of the recombinant plasmid DNA from *Escherichia coli* carrying the DNA is carried out by the method described in Example 1 or the method of Birnboim, et al. [H.C. Birnboim, et al.: Nucleic Acids Res. 7, 1513 (1979)]. Plasmid DNA is digested with 1 - 10 kinds of restriction endonucleases and the cleavage sites are examined by agarose gel electrophoresis or polyacrylamide gel electrophoresis. Further, if necessary, the sequence of the DNA is determined by the method of Maxam-Gilbert [Proc. Natl. Acad. Sci. 74, 560 (1977)].

The recombinant plasmids can be prepared under the above-described conditions.

The derivative of IFN- $\gamma$  polypeptide of the present invention is produced by the following method.

That is, *Escherichia coli* K-12 HB101 is transformed with a plasmid such as pGVA4 and an *Escherichia coli* strain carrying pGVA4 is selected from the ampicillin resistant (referred to as Ap<sup>R</sup> hereinafter) colonies. The *Escherichia coli* strain carrying pGVA4 is cultured in a medium to produce a derivative of IFN- $\gamma$  polypeptide in the medium.

As the medium, either a synthetic medium or a natural medium can be used as long as it is suitable for the growth of *Escherichia coli* and the production of the derivative of IFN- $\gamma$  polypeptide.

As a carbon source, glucose, fructose, lactose, glycerol, mannitol, sorbitol, etc. may be used.

As a nitrogen source, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, casamino acid, yeast extract, polypeptone, meat extract, Bacto-trypton, corn steep liquor, etc. may be used.

In addition, nutrients such as K<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, NaCl, MgSO<sub>4</sub>, vitamin B<sub>1</sub> and MgCl<sub>2</sub> may be used.

Culturing is carried out at pH 5.5 - 8.5 and at 18 - 40 °C with aeration and stirring.

After culturing for 5 - 90 hours, the derivative of human IFN- $\gamma$  polypeptide is accumulated in cultured cells. The collected cells are treated with lysozyme, disrupted by repeated freezing and thawing and subjected to centrifugation. The thus obtained supernatant fluid is subjected to extraction according to a conventional method for extraction of polypeptides to recover the polypeptide.

Determination of the human IFN- $\gamma$  activity is carried out according to the method of Armstrong [J.A. Armstrong, et al.: Appl. Microbiol. 21, 723 - 725 (1971)].

Certain specific embodiments of the present invention are illustrated by the following examples.

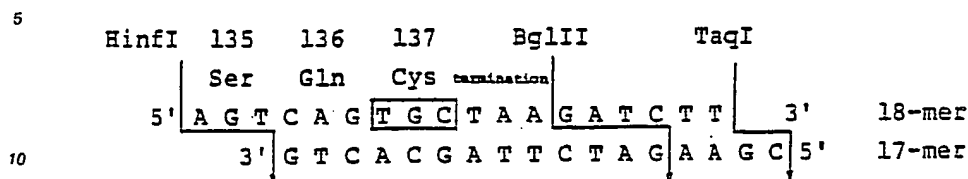
#### Example 1

Construction of recombinant plasmid pGVT137 coding for 137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146):

In this step, 10  $\mu$ g of pGBD1 DNA obtained in Reference Example 3 was dissolved in 50  $\mu$ l (total volume) of a solution consisting of 20 mM Tris-HC (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 100 mM NaCl (referred to as "Y-100 buffer solution" hereinafter). Then, 20 units of restriction enzyme BamHI (product of Takara Shuzo Co., the restriction enzymes hereinafter are all products of Takara Shuzo Co. unless otherwise specified) was added and digestion reaction was carried out at 37 °C for 3 hours. Subsequently, 20 units of restriction enzyme TaqI was added and digestion reaction was carried out at 65 °C for 2 hours. About 0.5  $\mu$ g of a DNA fragment of about 310 bp containing 3' non-translational region was obtained from the reaction solution by polyacrylamide gel electrophoresis.

Separately, 10  $\mu$ g of pGKA2 DNA obtained in Reference Example 2 was dissolved in 50  $\mu$ l (total volume) of Y-100 buffer solution and 20 units each of restriction enzymes HindIII and HinfI were added to the solution. Digestion reaction was carried out at 37 °C for 3 hours. A DNA fragment of about 400 bp containing a large portion of the IFN- $\gamma$  structural gene was obtained from the reaction solution by polyacrylamide gel electrophoresis. 3  $\mu$ g of pKYP10 DNA prepared by the method described in Japanese Published Unexamined Patent Application No. 110600/83 was dissolved in 40  $\mu$ l (total volume) of Y-100 buffer solution and 6 units each of restriction enzymes HindIII and BamHI were added to the solution. Digestion reaction was carried out at 37 °C for 3 hours. 1.8  $\mu$ g of a DNA fragment of about 4.3 Kb containing a tryptophan promoter (Ptrp) was obtained from the reaction solution by LGT method. Sepa-

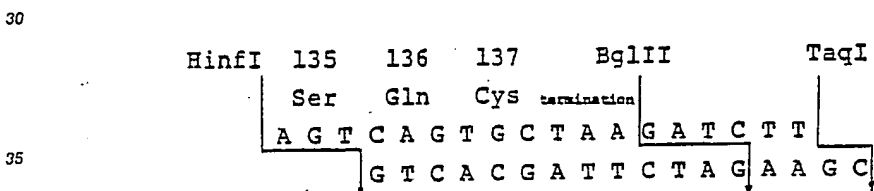
rately, in order to change the 137th amino acid (Met) of the mature human IFN- $\gamma$  polypeptide to Cys and to furnish a terminal codon (TAA) necessary for the termination of expression immediately after the 137th Cys, the following DNA linker was synthesized.



Two single chain DNAs of 18-mer and 17-mer were synthesized by a conventional triester method [R. Crea, et al.: Proc. Natl. Acad. Sci., USA, 75, 5765 (1978)]. Then, 2  $\mu$ g each of the 18-mer and 17-mer DNAs were dissolved in 40  $\mu$ l (total volume) of a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP. 30 units of T4 polynucleotide kinase (product of Takara Shuzo Co.) was added and phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.5  $\mu$ g of the TaqI-BamHI fragment of about 310 bp derived from pGBD1, 0.5  $\mu$ g of the HindIII-HinfI fragment of about 400 bp derived from pGKA2 and 1.0  $\mu$ g of the HindIII-BamHI fragment of about 4.3 Kb of the expression vector pKYP10 which were obtained above were dissolved in 25  $\mu$ l of T4 ligase buffer solution. About 0.1  $\mu$ g of the DNA linker mentioned above was added to the mixture, followed by addition of 6 units of T4 DNA ligase. Ligation reaction was carried out at 4°C for 17 hours.

Escherichia coli HB101 was transformed using the resulting recombinant plasmid mixture to obtain an Ap<sup>R</sup> colony. Plasmid pGVT137 illustrated in Fig. 1 was isolated from the culture broth of the colony. The structure of pGVT137 was confirmed by the digestion with EcoRI, ClaI, BglII and BamHI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert [A.M. Maxam, et al.: Proc. Natl. Acad. Sci., USA, 74, 560 (1977)] that the DNA sequence of the HinfI-TaqI fragment in the plasmid pGVT137 was as follows.



The human IFN- $\gamma$  polypeptide derivative encoded by pGVT137 [the derivative is named 137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146)] is clearly different from the known human IFN- $\gamma$  polypeptide in that the 137th amino acid (Met) of the mature human IFN- $\gamma$  is replaced with Cys and the 9 amino acids from the C-terminal 138th amino acid (Leu) to the 146th amino acid (Gln) are deleted.

Escherichia coli strain carrying plasmid pGVT137 has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology (referred to as FRI hereinafter) as Escherichia coli IGVT137 (FERM BP-547).

## Example 2

50 Construction of recombinant plasmid pGNC5 coding for 1-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146):

In this step, 10  $\mu$ g of pGVL10 DNA obtained in Reference Example 4 was dissolved in 50  $\mu$ l of Y-100 buffer solution. 20 units of restriction enzyme HindIII was added and digestion reaction was carried out at 37°C for 3 hours. Then, 20 units of restriction enzyme TagI was added and digestion reaction was carried out at 65°C for 2 hours. About 0.5  $\mu$ g of a DNA fragment of about 300 bp containing the 5' terminal region of the IFN- $\gamma$  structural gene was obtained from the reaction solution by polyacrylamide gel electrophoresis.

Separately, 10  $\mu$ g of pGVT137 DNA obtained in Example 1 was dissolved in 50  $\mu$ l of Y-100 buffer

solution and 20 units of restriction enzyme BglII was added. Digestion reaction was carried out at 37 °C for 3 hours. Then, 20 units of restriction enzyme TagI was added and digestion reaction was carried out at 65 °C for 2 hours. About 0.4 µg of a DNA fragment of about 130 bp containing the 3' terminal region of the IFN-γ structural gene was obtained from the reaction solution by polyacrylamide gel electrophoresis.

5 Separately, 5  $\mu$ g of plasmid pGVT137 DNA was dissolved in 50  $\mu$ l of Y-100 buffer solution and 10 units each of restriction enzymes HindIII and BglII were added. Digestion reaction was carried out at 37°C for 3 hours.

About 2.0  $\mu$ g of a DNA fragment of about 4.6 Kb containing the 3' non-translational region and P<sub>trp</sub> was obtained from the reaction solution by LGT method.

10 Then, 0.4  $\mu$ g of the HindIII-TagI fragment (about 300 bp) derived from pGVL10, 0.5  $\mu$ g of the BglII-TagI fragment (about 130 bp) derived from pGVT137 and 1.0  $\mu$ g of the HindIII-BglII fragment (about 4.6 Kb) derived from the same plasmid which were obtained above were dissolved in 25  $\mu$ l of T4 DNA ligase buffer solution. 6 units of T4 DNA ligase was added and ligation reaction was carried out at 4  $^{\circ}$ C for 17 hours.

Escherichia coli HB101 strain was transformed with the resulting recombinant plasmid mixture to obtain an Ap<sup>R</sup> colony.

Plasmid pGNC5 illustrated in Fig. 2 was isolated from the culture broth of the colony. The structure of pGNC5 was confirmed by the digestion with HindIII, EcoRI, BglII, ClaI and BamHI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert that the DNA sequence of the HindIII-SinI fragment in pGNC5 was as follows:

20

SinI

HindIII		Met	Ser	Tyr	Cys	Gln	Asp
	A	G	C	T	T	A	T
	G	T	C	T	T	A	C
	T	G	T	C	T	T	A
	C	T	T	A	C	T	G
	C	C	A	G	G	A	C

25

and the DNA sequence of the *Hinf*I-*Taq*I fragment was as follows:

```

30      HinfI                               BglIII           TaqI
       |         135     136     137          |               |
       |   Ser   Gln   Cys termination    |               |
       | A G T C A G T G C T A A | G A T C T T | C G
       |                           |               |
25   +-----+                   +-----+

```

The human IFN- $\gamma$  polypeptide derivative encoded by pGNC5 [the derivative is named 1-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146)] is clearly different from the known human IFN- $\gamma$  polypeptide in that the first amino acid (Cys) of the mature human IFN- $\gamma$  is replaced with Ser, the 137th amino acid (Met) is replaced with Cys and the 9 amino acids from the C-terminal 138th amino acid (Leu) to 146th amino acid (Gln) are deleted. *Escherichia coli* strain carrying plasmid pGNC5 has been deposited with FRI as *Escherichia coli* IGNC5 (FERM BP-550).

45 Example 3

**Construction of recombinant plasmid pGNB4 coding for 3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146):**

50 In this step, 10  $\mu$ g of pGVM101 DNA obtained in Reference Example 5 was dissolved in 50  $\mu$ l of Y-100 buffer solution. 20 units of restriction enzyme HindIII was added and digestion reaction was carried out at 37°C for 3 hours. Then, 20 units of restriction enzyme TaqI was added and digestion reaction was carried out at 65°C for 2 hours. About 0.5  $\mu$ g of a DNA fragment of about 300 bp containing the 5' terminal region of the IFN- $\gamma$  structural gene was obtained from the reaction solution by polyacrylamide gel electrophoresis.

55 Separately, 10  $\mu$ g of pGVT137 DNA obtained in Example 1 was dissolved in 50  $\mu$ l of Y-100 buffer solution and 20 units of restriction enzyme BglII was added. Digestion reaction was carried out at 37 $^{\circ}$ C for 3 hours. Then, 20 units of restriction enzyme TagI was added and digestion reaction was carried out at 65 $^{\circ}$ C for 2 hours. About 0.4  $\mu$ g of a DNA fragment of about 130 bp containing the 3' terminal region of the

IFN- $\gamma$  structural gene was obtained from the reaction solution by polyacrylamide gel electrophoresis.

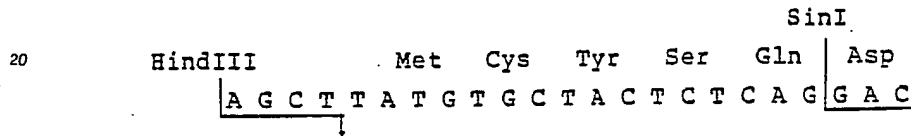
Separately, 5  $\mu$ g of plasmid pGVT137 DNA was dissolved in 50  $\mu$ l of Y-100 buffer solution and 10 units each of restriction enzymes HindIII and BglII were added. Digestion reaction was carried out at 37°C for 3 hours.

5 About 2.0  $\mu$ g of a DNA fragment of about 4.6 Kb containing the 3' non-translational region and P<sub>trp</sub> was obtained from the reaction solution by LGT method.

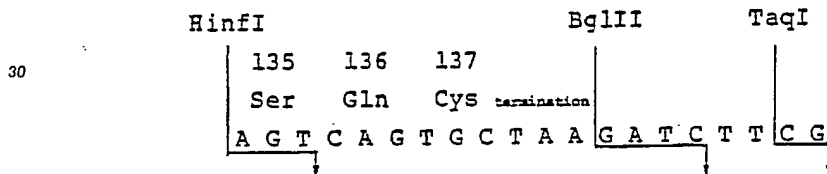
Then, 0.4 g of the HindIII-TaqI fragment (about 300 bp) derived from pGVM101, 0.5  $\mu$ g of the BglII-TaqI fragment (about 130 bp) derived from pGVT137 and 1.0  $\mu$ g of the HindIII-BglII fragment (about 4.6 Kb) derived from the same plasmid which were obtained above were dissolved in 25  $\mu$ l of T4 DNA ligase buffer solution. 6 units of T4 DNA ligase was added and ligation reaction was carried out at 4 °C for 17 hours.

Escherichia coli HB101 strain was transformed with the resulting recombinant plasmid mixture to obtain an Ap<sup>R</sup> colony.

Plasmid pGNB4 illustrated in Fig. 3 was isolated from the culture broth of the colony. The structure of pGNB4 was confirmed by the digestion with HindIII, EcoRI, BglII, ClaI and BamHI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert that the DNA sequence of the HindIII-SinI 15 fragment in pGNB4 was as follows:



25 and the DNA sequence of the *Hinf*I-*Taq*I fragment was as follows:



The human IFN- $\gamma$  polypeptide derivative encoded by pGNB4 [the derivative is named 3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146)] is clearly different from the known human IFN- $\gamma$  polypeptide in that the third amino acid (Cys) of the mature human IFN- $\gamma$  is replaced with Ser, the 137th amino acid (Met) is replaced with Cys and the 9 amino acids from the C-terminal 138th amino acid (Leu) to 146th amino acid (Gln) are deleted.

40 The *Escherichia coli* strain carrying plasmid pGNB4 has been deposited with FRI as *Escherichia coli* IGBN4 (FERM BP-549).

### Example 4

#### Construction of recombinant plasmid pGNA3 coding for 1,3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146):

In this step, 10  $\mu$ g of pGVK13 DNA obtained in Reference Example 6 was dissolved in 50  $\mu$ l of Y-100 50 buffer solution. 20 units of restriction enzyme HindIII was added and digestion reaction was carried out at 37 $^{\circ}$ C for 3 hours. Then, 20 units of restriction enzyme TagI was added and digestion reaction was carried out at 65 $^{\circ}$ C for 2 hours. About 0.5  $\mu$ g of a DNA fragment of about 300 bp containing the 5' terminal region of the IFN- $\gamma$  structural gene was obtained from the reaction solution by polyacrylamide gel electrophoresis.

Separately, 10  $\mu$ g of pGVT137 DNA obtained in Example 1 was dissolved in 50  $\mu$ l of Y-100 buffer  
55 solution and 20 units of restriction enzyme BglII was added. Digestion reaction was carried out at 37 $^{\circ}$  C for  
3 hours. Then, 20 units of restriction enzyme TagI was added and digestion reaction was carried out at  
65 $^{\circ}$  C for 2 hours. 0.4  $\mu$ g of a DNA fragment of about 130 bp containing the 3' terminal region of the IFN- $\gamma$   
structural gene was obtained from the reaction solution by polyacrylamide gel electrophoresis.



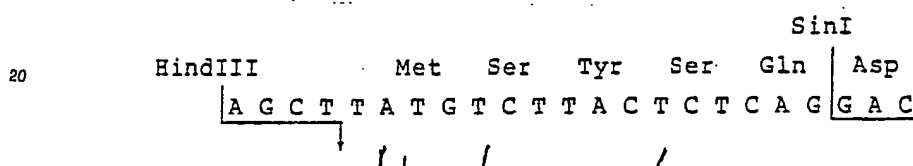
Separately, 5  $\mu$ g of plasmid pGVT137 DNA was dissolved in 50  $\mu$ l of Y-100 buffer solution and 10 units each of restriction enzymes HindIII and BglII were added. Digestion reaction was carried out at 37° C for 3 hours.

About 2.0  $\mu$ g of a DNA fragment of about 4.6 Kb containing the 3' non-translational region and P<sub>trp</sub> was obtained from the reaction solution by LGT method.

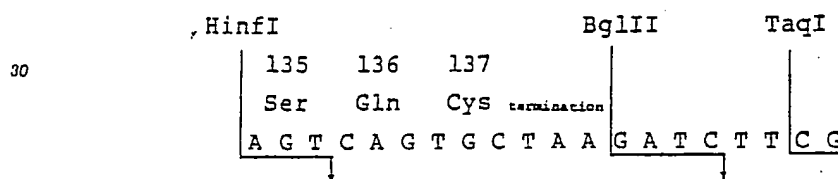
Then, 0.4  $\mu$ g of the HindIII-TaqI fragment (about 300 bp) derived from pGVK13, 0.5  $\mu$ g of the BglII-TagI fragment (about 130 bp) derived from pGVT137 and 1.0  $\mu$ g of the HindIII-BglII fragment (about 4.6 Kb) derived from the same plasmid which were obtained above were dissolved in 25  $\mu$ l of T4 DNA ligase buffer solution. 6 units of T4 DNA ligase was added and ligation reaction was carried out at 4° C for 17 hours.

*Escherichia coli* HB101 strain was transformed with the resulting recombinant plasmid mixture to obtain an Ap<sup>R</sup> colony.

Plasmid pGNA3 illustrated in Fig. 4 was isolated from the culture broth of the colony. The structure of pGNA3 was confirmed by the digestion with HindIII, EcoRI, BglII, ClaI and BamHI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert that the DNA sequence of the HindIII-SinI fragment in pGNA3 was as follows:



and the DNA sequence of the HinfI-TaqI fragment was as follows:



The human IFN- $\gamma$  polypeptide derivative encoded by pGNA3 [the derivative is named 1,3-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146)] is clearly different from the known human IFN- $\gamma$  polypeptide in that the first and third amino acids (Cys) of the mature human IFN- $\gamma$  are replaced with Ser, the 137th amino acid (Met) is replaced with Cys and the 9 amino acids from the C-terminal 138th amino acid (Leu) to 146th amino acid (Gln) are deleted. *Escherichia coli* strain carrying plasmid pGNA3 has been deposited with FRI as *Escherichia coli* IGNA3 (FERM BP-548).

#### Example 5

Production of IFN- $\gamma$  derivatives by *Escherichia coli* strains carrying pGVT137, pGNC5, pGNB4 and pGNA3:

*Escherichia coli* HB101 strains carrying recombinant plasmids pGVT137, pGNC5, pGNB4 and pGNA3 obtained in Examples 1 - 4, which are named IGVT137, IGNC5, IGNB4 and IGNA3 respectively, were cultured at 37° C for 18 hours in LG medium (10 g of trypton, 5 g of yeast extract, 5 g of NaCl, 2 g of glucose, 1 l of water, adjusted to pH 7.0 with NaOH). 0.2 ml of the culture broth was inoculated into 10 ml of MCG medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.5% NaCl, 0.1% NH<sub>4</sub>Cl, 0.5% glucose, 0.5% casamino acid, 1 mM MgSO<sub>4</sub>, 4  $\mu$ g/ml vitamin B<sub>1</sub>, pH 7.2) and culturing was carried out at 30° C for 4 - 8 hours. Then, 10  $\mu$ g/ml indolylacrylic acid (referred to as IAA hereinafter) which is an inducer of tryptophan was added and culturing was continued for 2 - 12 hours. The culture broth was centrifuged at 8,000 rpm for 10 minutes and the cells harvested were washed with a buffer solution containing 30 mM NaCl and 30 mM Tris-HCl (pH 7.5). Washed cells were suspended in 1 ml of the buffer solution described above and 5  $\mu$ l of

a solution containing 200  $\mu$ g of lysozyme and 0.25M EDTA (ethylenediamine tetraacetic acid) was added. The mixture was allowed to stand at 0°C for 30 minutes and freezing and thawing were repeated three times to disrupt the cells. The disrupted cells were centrifuged at 15,000 rpm for 30 minutes to obtain a supernatant fluid. The amount of interferon in the supernatant was determined according to the method of Armstrong [J.A. Armstrong, et al.: Appl. Microbiol. 21, 723 - 725 (1971)], wherein Sindbis virus was used as the virus and FL cells derived from human amnion cells were used as the animal cells. The results are shown in Table 2.

Table 2

Strain	Plasmid	Product encoded by the plasmid	IFN- $\gamma$ (units/ml)
IGVT137	pGVT137	137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146)	8 x 10 <sup>4</sup>
IGNA3	pGNA3	1,3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146)	8 x 10 <sup>5</sup>
IGNB4	pGNB4	3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146)	1 x 10 <sup>6</sup>
IGNC5	pGNC5	1-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146)	5 x 10 <sup>5</sup>
IGKA2	pGKA2	IFN- $\gamma$	2 x 10 <sup>4</sup>

IGKA2 is a strain carrying plasmid pGKA2 coding for IFN- $\gamma$ .

#### Example 6

Experiment on heat stability of IFN- $\gamma$  derivatives; 137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146), 1-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146), 3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146) and 1,3-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146):

*Escherichia coli* HB101 strains carrying recombinant plasmids pGVT137, pGNC5, pGNB4 and pGNA3 obtained in Examples 1 - 4, which are named IGVT137, IGNC5, IGNB4 and IGNA3 respectively, were cultured at 37°C for 18 hours in LG medium. 0.2 ml of the culture broth was inoculated into 10 ml of MCG medium and culturing was carried out at 30°C for 4 - 8 hours. Then, 10  $\mu$ g/ml IAA was added and culturing was continued for 2 - 12 hours. The culture broth was centrifuged at 8,000 rpm for 10 minutes and the cells harvested were washed with 30 mM Tris-HCl (pH 7.5) buffer solution containing 30 mM NaCl. Washed cells were suspended in 1 ml of the buffer solution described above and 5  $\mu$ l of a solution containing 200  $\mu$ g of lysozyme and 0.25M EDTA was added. The mixture was allowed to stand at 0°C for 30 minutes and freezing and thawing were repeated three times to disrupt the cells. The disrupted cells were centrifuged at 15,000 rpm for 30 minutes to obtain a supernatant fluid.

The supernatant was heated at 50°C for one hour and the anti-viral activity of interferon was determined according to the method of Armstrong to measure the residual activity (%).

The results are shown in Table 3.

Table 3

5	Strain	Plasmid	Product encoded by the plasmid	Residual activity after heat treatment at 50°C for 1 hour (%)
10	IGVT137	pGVT137	137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146)	81
	IGNC5	pGNC5	1-Ser-137-Cys- IFN- $\gamma$ ( $\Delta$ 138-146)	86
15	IGNB4	pGNB4	3-Ser-137-Cys- IFN- $\gamma$ ( $\Delta$ 138-146)	98
	IGNA3	pGNA3	1,3-Ser-137-Cys- IFN- $\gamma$ ( $\Delta$ 138-146)	23
20	IGKA2	pGKA2	IFN- $\gamma$	25

25

Reference Example 130 Insertion of human IFN- $\gamma$  DNA into the expression vector pKYP11:

In this example, 6  $\mu$ g of plasmid pIFN- $\gamma$ -G4 (3.6 Kb) was dissolved in 50  $\mu$ l (total volume) of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 50 mM NaCl. Then, 12 units each of restriction enzymes PvuII and HindIII were added and digestion reaction was carried out at 37°C for 4 hours. The reaction solution was heated at 65°C for 7 minutes to inactivate the enzymes and subjected to purification by LGT method to obtain 1.2  $\mu$ g of a DNA fragment of 1.3 Kb containing human IFN- $\gamma$  DNA.

Separately, 4  $\mu$ g of pKYP11 was dissolved in 40  $\mu$ l (total volume) of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 50 mM NaCl. 8 units of BamHI was added and digestion reaction was carried out at 37°C for 3 hours. The reaction solution was heated at 65°C for 5 minutes to inactivate the enzyme. Thereafter, 30  $\mu$ M each of dATP, dCTP, dGTP and dTTP were added and 8 units of *Escherichia coli* DNA polymerase I (Klenow fragment, product of New England Biolabs, 1  $\mu$ l) was added. Fill-in reaction was carried out at 15°C for one hour and the reaction solution was heated at 68°C for 15 minutes to inactivate DNA polymerase I. 10 units of HindIII was added and digestion reaction was carried out at 37°C for 3 hours, followed by heating at 65°C for 5 minutes to inactivate HindIII. The digestion reaction solution of the plasmid pKYP11 was subjected to purification by LGT method to obtain about 2.5  $\mu$ g of a DNA fragment of about 4.7 Kb containing P<sub>trp</sub>.

Then, 0.5  $\mu$ g of the DNA fragment of 1.3 Kb containing human IFN- $\gamma$  DNA and 1.0  $\mu$ g of the DNA fragment of about 4.7 Kb containing P<sub>trp</sub>, which was obtained from the plasmid pKYP11, were dissolved in 20  $\mu$ l of a solution containing 20 mM Tris-HCl (pH 7.5), 6 mM MgCl<sub>2</sub>, 5 mM dithiothreitol and 500  $\mu$ M ATP, and 4 units of T4 DNA ligase was added. Ligation reaction was carried out at 4°C for 18 hours, and *Escherichia coli* HB101 was transformed with the resulting recombinant plasmid mixture by conventional technique to obtain an Ap<sup>R</sup> colony. Plasmid pGC7 was separated from the culture broth of the colony. The structure of pGC7 was confirmed by the digestion with HindIII, HpaI, SalI, EcoRI and ClaI and agarose gel electrophoresis. *Escherichia coli* strain containing pGC7 has been deposited with FRI as *Escherichia coli* IGC7 (FERM BP-497).

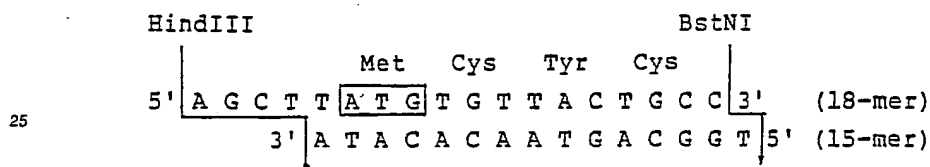
Reference Example 2

#### Construction of recombinant plasmid pGKA2:

In this example, 6  $\mu$ g of the pGC7 DNA obtained in Reference Example 1 was dissolved in 50  $\mu$ l (total volume) of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 10 mM NaCl, and 12 units of BstNI (product of New England Biolabs) was added. Reaction was carried out at 60 °C for 3 hours, and the reaction solution was heated at 65 °C for 5 minutes to inactivate BstNI. Then, NaCl was added to a final concentration of 150 mM and 8 units of Sall was added. Digestion reaction was carried out at 37 °C for 3 hours. The reaction solution was again heated at 65 °C for 5 minutes to inactivate Sall and subjected to purification by LGT method to obtain about 0.8  $\mu$ g of a DNA fragment of about 1,125 bp containing a large portion of the human IFN- $\gamma$  DNA.

Separately, 3  $\mu$ g of pKYP10 was dissolved in 40  $\mu$ l (total volume) of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM  $MgCl_2$ , 10 mM dithiothreitol and 100 mM NaCl. 6 units each of HindIII and Sall were added and digestion reaction was carried out at 37°C for 3 hours. The reaction solution was heated at 65°C for 5 minutes to inactivate HindIII and Sall and subjected to purification by LGT method to obtain about 1.8  $\mu$ g of a DNA fragment of about 4.1 Kb containing  $p_{Tet}$ .

The N-terminal amino acid of the mature human IFN- $\gamma$  polypeptide is Cys. In order to express mature IFN- $\gamma$  DNA, it is necessary to furnish an initiation codon (ATG) just before the 5'-terminal codon TGT (Cys) and further to adjust the length between SD-sequence downstream from P<sub>trp</sub> and ATG to a suitable length of 6-18 bp. Therefore, the following DNA linker was synthesized.



Two single chain DNAs of 18-mer and 15-mer were synthesized by a conventional triester method. Then, 2  $\mu$ g each of the 18-mer and 15-mer DNAs were dissolved in 20  $\mu$ l (total volume) of a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM  $MgCl_2$ , 5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP. 30 units of T4 polynucleotide kinase (product of Boehringer Mannheim GmbH) was added and phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 2  $\mu$ g each of phosphorylated 18-mer and 15-mer DNAs were mixed and the mixture was heated  
35 at 70 °C for 5 minutes and allowed to stand at room temperature for annealing to obtain the DNA linker  
having the structure given above:

0.4  $\mu$ g of the BstNI-Sall fragment of 1,125 bp obtained above and derived from pGC7 and 1.0  $\mu$ g of the DNA fragment of 4.1 Kb obtained by digestion of the expression vector pKYP10 with HindIII and Sall were dissolved in 25  $\mu$ l (total volume) of a solution containing 20 mM Tris-HCl (pH 7.5), 6 mM MgCl<sub>2</sub>, 5 mM dithiothreitol and 500  $\mu$ M ATP. About 0.1  $\mu$ g of the DNA linker mentioned above was added to the mixture, followed by addition of 6 units of T4 DNA ligase. Ligation reaction was carried out at 4 °C for 17 hours. *Escherichia coli* HB101 was transformed using the resulting recombinant plasmid mixture by conventional technique to obtain an Ap<sup>R</sup> colony. A plasmid, pGKA2 illustrated in Fig. 1 was isolated from the culture broth of the colony. The structure of pGKA2 was confirmed by the digestion with EcoRI, ClaI, HindIII, BstNI and Sall and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert that the DNA sequence from the SD-sequence (AAGG) to the initiation codon (ATG) in the plasmid pGKA2 was "AAGGGTATCGATAAGCTTATG".

Escherichia coli strain containing pGKA2 has been deposited with FRI as Escherichia coli IGKA2 (FERM BP-496).

### Reference Example 3

55 Construction of plasmid pGBD1 having BamHI cleavage site downstream from IFN- $\gamma$  gene:

In this example, 2  $\mu$ g of plasmid pIFN $\gamma$ -G4 (3.6 Kb) was dissolved in 50  $\mu$ l (total volume) of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 50 mM NaCl (referred to as

"Y-50 buffer solution" hereinafter). Then, 4 units of restriction enzyme PvuII was added and digestion reaction was carried out at 37°C for 2 hours. The digest was subjected to purification by LGT method to obtain 1 µg of a DNA fragment (3.6 Kb) of pIFN-γ-G4. 0.1 µg of the DNA fragment and 5 pmoles of 5'-phosphorylated BamHI linker (5'-pCCGGATCCGG-3': product of Collaborative Research, Inc.) were ligated at 4°C for 18 hours with 2 units of T4 DNA ligase in 20 µl of T4 DNA ligase buffer solution.

*Escherichia coli* HB101 [Bolivar, et al.: GENE 2, 75 (1977)] was transformed using the thus obtained recombinant plasmid DNA by the method of Cohen, et al. [S.N. Cohen, et al.: Proc. Natl. Acad. Sci. USA. 69, 2110 (1972)] to obtain an Ap<sup>R</sup> colony. Plasmid DNA was isolated from the transformant by the known method [H.C. Birnboim, et al: Nucleic Acids Res., 7, 1513 (1979)]. The DNA was digested with restriction endonucleases such as BamHI and its structure was analyzed to recognize that recombinant plasmid pGBD1 wherein BamHI linker was inserted into PvuII site of pIFN-γ-G4 was obtained. *Escherichia coli* strain carrying plasmid pGBD1 has been deposited with FRI as *Escherichia coli* IGBD1 (FEB BP-394).

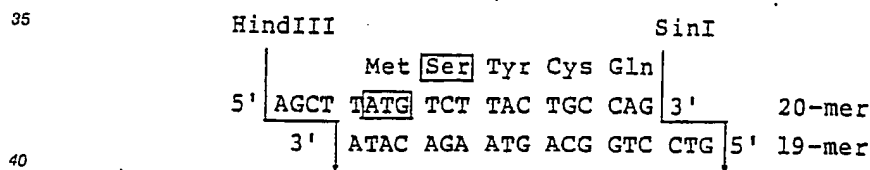
#### 15 Reference Example 4

Construction of recombinant plasmid pGVL10 coding for 1-Ser-IFN-γ:

20 In this example, 6 µg of pGBD1 DNA obtained in Reference Example 3 was dissolved in 50 µl of Y-50 buffer solution. 10 units of SmaI was added and digestion reaction was carried out at 37°C for 3 hours. Then, NaCl was added to a final concentration of 100 mM and 10 units of BamHI was added. Digestion reaction was carried out at 37°C for 3 hours. 0.8 µg of a DNA fragment of about 850 bp containing a large portion of the human IFN-γ DNA was obtained from the reaction solution by LGT method.

25 Separately, 3 µg of pKYP10 DNA prepared by the method described in Japanese Published Unexamined Patent Application No. 110600/83 was dissolved in 40 µl (total volume) of Y-50 buffer solution and 5 units each of HindIII and BamHI were added. Digestion reaction was carried out at 37°C for 3 hours. From the reaction solution, about 1.8 µg of a DNA fragment of about 4.3 Kb containing tryptophan promoter (P<sub>trp</sub>) was obtained by LGT method.

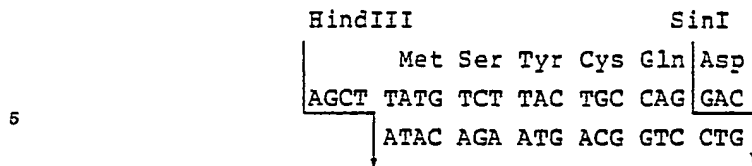
30 Mature human IFN-γ polypeptide has the N-terminal structure of Cys-Tyr-Cys-. In order to change the first Cys to Ser and to furnish an initiation codon (ATG) necessary for expression just before the first Se, the following DNA linker was synthesized.



Two single chain DNAs of 20-mer and 19-mer were synthesized by a conventional triester method. Then, 2 µg each of the 20-mer and 19-mer DNAs were dissolved in 40 µl (total volume) of a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP. 30 units of T4 polynucleotide kinase was added and phosphorylation reaction was carried out at 37°C for 60 minutes.

0.5 µg of the SmaI-BamHI fragment of about 850 bp derived from pGBD1 and 1.0 µg of the HindIII-BamHI fragment of about 4.3 Kb of the expression vector pKYP10 which were obtained above were dissolved in 25 µl of T4 DNA ligase buffer solution. About 0.1 µg of the DNA linker mentioned above was added to the mixture, followed by addition of 6 units of T4 DNA ligase. Ligation reaction was carried out at 4°C for 17 hours.

*Escherichia coli* HB101 was transformed using the resulting recombinant plasmid mixture to obtain an Ap<sup>R</sup> colony. A plasmid, pGVL10 illustrated in Fig. 2 was isolated from the culture broth of the colony. The structure of pGVL10 was confirmed by the digestion with EcoRI, ClaI, HindIII and BamHI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert that the DNA sequence from the HindIII site to the SmaI site in the plasmid pGVL10 was as follows.



10 The human IFN- $\gamma$  polypeptide encoded by pGVL10 (the derivative is named 1-Ser-IFN- $\gamma$ ) is clearly different from the known human IFN- $\gamma$  polypeptide in that the first Cys of the mature human IFN- $\gamma$  is replaced with Ser. *Escherichia coli* strain carrying plasmid pGVL10 has been deposited with FRI as *Escherichia coli* IGV10 (FERM BP-544).

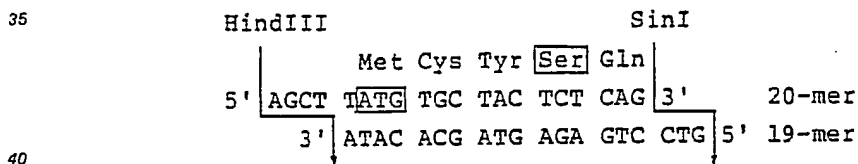
#### 15 Reference Example 5

Construction of recombinant plasmid pGVM101 coding for 3-Ser-IFN- $\gamma$ :

20 In this example, 6  $\mu$ g of pGBD1 DNA obtained in Reference Example 3 was dissolved in 50  $\mu$ l of Y-50 buffer solution. 10 units of SinI was added and digestion reaction was carried out at 37°C for 3 hours. Then, NaCl was added to a final concentration of 100 mM and 10 units of BamHI was added. Digestion reaction was carried out at 37°C for 3 hours. 0.8  $\mu$ g of a DNA fragment of about 850 bp containing a large portion of the human IFN- $\gamma$  DNA was obtained from the reaction solution by LGT method.

25 Separately, 3  $\mu$ g of pKYP10 DNA prepared by the method described in Japanese Published Unexamined Patent Application No. 110600/83 was dissolved in 40  $\mu$ l (total volume) of Y-50 buffer solution and 5 units each of HindIII and BamHI were added. Digestion reaction was carried out at 37°C for 3 hours. From the reaction solution, about 1.8  $\mu$ g of a DNA fragment of about 4.3 Kb containing tryptophan promoter ( $P_{trp}$ ) was obtained by LGT method.

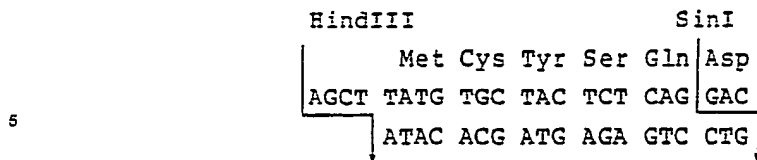
30 Mature human IFN- $\gamma$  polypeptide has the N-terminal structure of Cys-Tyr-Cys-. In order to change the third amino acid (Cys) to Ser and to furnish an initiation codon (ATG) necessary for expression just before the first Cys, the following DNA linker was synthesized.



Two single chain DNAs of 20-mer and 19-mer were synthesized by a conventional triester method. Then, 2  $\mu$ g each of the 20-mer and 19-mer DNAs were dissolved in 40  $\mu$ l (total volume) of a solution 45 containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP. 30 units of T4 polynucleotide kinase was added and phosphorylation reaction was carried out at 37°C for 60 minutes.

0.5  $\mu$ g of the SinI-BamHI fragment of about 850 bp derived from pGBD1 and 1.0  $\mu$ g of the HindIII-BamHI fragment of about 4.3 Kb of the expression vector pKYP10 which were obtained above were 50 dissolved in 25  $\mu$ l of T4 DNA ligase buffer solution. About 0.1  $\mu$ g of the DNA linker mentioned above was added to the mixture, followed by addition of 6 units of T4 DNA ligase. Ligation reaction was carried out at 4°C for 17 hours.

*Escherichia coli* HB101 was transformed using the resulting recombinant plasmid mixture to obtain an Ap<sup>R</sup> colony. A plasmid, pGVM101 illustrated in Fig. 3 was isolated from the culture broth of the colony. The 55 structure of pGVM101 was confirmed by the digestion with EcoRI, ClaI, HindIII and BamHI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert that the DNA sequence from the HindIII site to the SinI site in the plasmid pGVM101 was as follows.



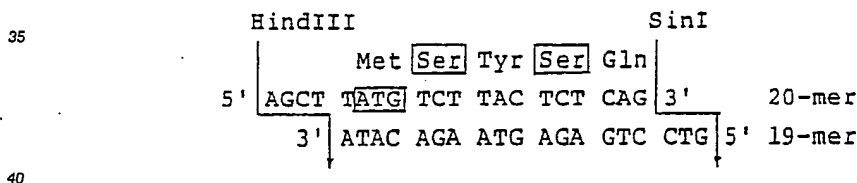
10 The human IFN- $\gamma$  polypeptide encoded by pGVM101 (the derivative is named 3-Ser-IFN- $\gamma$ ) is clearly different from the known human IFN- $\gamma$  polypeptide in that the third amino acid (Cys) of the mature human IFN- $\gamma$  is replaced with Ser. *Escherichia coli* strain carrying plasmid pGVM101 has been deposited with FRI as *Escherichia coli* IGVM101 (FERM BP-545).

#### 15 Reference Example 6

Construction of recombinant plasmid pGVK13 coding for 1,3-Ser-IFN- $\gamma$ :

20 In this example, 6  $\mu$ g of pGBD1 DNA obtained in Reference Example 3 was dissolved in 50  $\mu$ l of Y-50 buffer solution. 10 units of SinI (product of Bio Tec Co.) was added and digestion reaction was carried out at 37°C for 3 hours. Then, NaCl was added to a final concentration of 100 mM and 10 units of BamHI was added. Digestion reaction was carried out at 37°C for 3 hours. About 0.8  $\mu$ g of a DNA fragment of about 850 bp containing a large portion of the human IFN- $\gamma$  DNA was obtained from the reaction solution by LGT method. Separately, 3  $\mu$ g of pKYP10 was dissolved in 40  $\mu$ l (total volume) of Y-100 buffer solution and 5 units each of HindIII and BamHI were added. Digestion reaction was carried out at 37°C for 3 hours. From the reaction solution, about 1.8  $\mu$ g of a DNA fragment of about 4.3 Kb containing P<sub>trp</sub> was obtained by LGT method.

30 Mature human IFN- $\gamma$  polypeptide has the N-terminal structure of Cys-Tyr-Cys-. In order to change the first and third amino acids (Cys) to Ser and to furnish an initiation codon (ATG) necessary for expression just before the first Ser, the following DNA linker was synthesized.



Two single chain DNAs of 20-mer and 19-mer were synthesized by a conventional triester method. Then, 2  $\mu$ g each of the 20-mer and 19-mer DNAs were dissolved in 40  $\mu$ l (total volume) of a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM EDTA and 1 mM ATP. 30 units of T4 polynucleotide kinase was added and phosphorylation reaction was carried out at 37°C for 60 minutes.

Then, 0.4  $\mu$ g of the SinI-BamHI fragment of about 850 bp derived from pGBD1 and 1.0  $\mu$ g of the HindIII-BamHI fragment of about 4.3 Kb of the expression vector pKYP10 which were obtained above were dissolved in 25  $\mu$ l of T4 DNA ligase buffer solution. About 0.1  $\mu$ g of the DNA linker mentioned above was added to the mixture, followed by addition of 6 units of T4 DNA ligase. Ligation reaction was carried out at 4°C for 17 hours.

55 *Escherichia coli* HB101 was transformed using the resulting recombinant plasmid mixture to obtain an Ap<sup>R</sup> colony. A plasmid, pGVK13 illustrated in Fig. 4 was isolated from the culture broth of the colony. The structure of pGVK13 was confirmed by the digestion with EcoRI, HindIII, ClaI and BamHI and agarose gel electrophoresis. It was confirmed by the method of Maxam-Gilbert that the DNA sequence from the HindIII site to the SinI site in the plasmid pGVR13 was as follows.

[illegible]

The human IFN- $\gamma$  polypeptide encoded by pGVK13 (the derivative is named 1,3-Ser-IFN- $\gamma$ ) is clearly different from the known human IFN- $\gamma$  polypeptide in that the first and third amino acids (Cys) of the mature human IFN- $\gamma$  are replaced with Ser. *Escherichia coli* strain carrying plasmid pGVK13 has been deposited with FRI as *Escherichia coli* IGVK13 (FERM BP-432).

## 15 Claims

1. Derivatives of a human interferon- $\gamma$  polypeptide having the formula. 137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146), 1-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146 ), or 3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146), and being encoded by the recombinant plasmid pGVT137 (FERM BP-547), pGNC5 (TERM BP-550) and pGNB4 (FERM BP-549), respectively.
2. The recombinant plasmid pGVT137 (FERM BP-547), pGNC5 (TERM BP-550) or pGNB4 (FERM BP-549).
3. A microorganism transformed with a recombinant plasmid according to Claim 2 which is Escherichia coli IGVT137 (FERM BP-547), Escherichia coli IGNC5 (FERM BP-550) or Escherichia coli IGNB4 (FERM BP-549).
4. A process for producing a derivative of a human interferon- $\gamma$  polypeptide according to Claim 1 which comprises culturing in a medium a microorganism according to Claim 3, said microorganism being capable of expressing the incorporated DNA fragment, accumulating said polypeptide in the culture medium and recovering said polypeptide therefrom.

Claims for the following Contracting State: AT

- 35 1. The recombinant plasmid pGVT137 (FERM BP-547), pGNC5 (TERM BP-550) or pGNB4 (FERM BP-549).
2. A microorganism transformed with a recombinant plasmid according to Claim 2 which is Escherichia coli IGV137 (FERM BP-547), Escherichia coli IGNC5 (FERM BP-550) or Escherichia coli IGNB4 (FERM BP-549).
- 40 3. A process for producing a derivative of a human interferon- $\gamma$  polypeptide having the formula 137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146), 1-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146), or 3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146), and being encoded by the recombinant plasmid pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) and pGNB4 (FERM BP-549), respectively, said process comprising culturing in a medium a microorganism according to Claim 2, said microorganism being capable of expressing the incorporated DNA fragment, accumulating said polypeptide in the culture medium and recovering said polypeptide therefrom.
- 45

## 50 Revendications

1. Dérivés de polypeptide interféron- $\gamma$  humain ayant la formule 137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146), 1-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146) ou 3-Ser-137-Cys-IFN- $\gamma$ ( $\Delta$ 138-146) et codés respectivement par le plasmide recombinant pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) ou pGNB4 (FERM BP-549).
2. Plasmide recombinant pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) ou pGNB4 (FERM BP-549).
3. Microorganisme transformé par un plasmide recombinant conforme à la revendication 2, qui est



Escherichia coli IGVT137 (FERM BP-547), Escherichia coli IGNC5 (FERM BP-550) ou Escherichia coli IGNB4 (FERM BP-549).

4. Procédé de production d'un dérivé de polypeptide interféron- $\gamma$  humain conforme à la revendication 1, qui comprend la culture, dans un milieu, d'un microorganisme conforme à la revendication 3, ledit microorganisme étant capable d'exprimer le fragment d'ADN incorporé, l'accumulation dudit polypeptide dans le milieu de culture et la récupération dudit polypeptide à partir de celui-ci.

Revendications pour l'Etat contractant suivant: AT

10

1. Plasmide recombinant pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) ou pGNB4 (FERM BP-549).

2. Microorganisme transformé par un plasmide recombinant conforme à la revendication 2, qui est Escherichia coli IGVT137 (FERM BP-547), Escherichia coli IGNC5 (FERM BP-550) ou Escherichia coli IGNB4 (FERM BP-549).

3. Procédé de production d'un dérivé de polypeptide interféron- $\gamma$  humain ayant la formule 137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146), 1-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146) ou 3-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146) et codé respectivement par le plasmide recombinant pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) ou pGNB4 (FERM BP-549), ledit procédé comprenant la culture, dans un milieu, d'un microorganisme conforme à la revendication 2, ledit microorganisme étant capable d'exprimer le fragment d'ADN incorporé, l'accumulation dudit polypeptide dans le milieu de culture et la récupération dudit polypeptide à partir de celui-ci.

25

#### Ansprüche

1. Derivate eines menschlichen Interferon- $\gamma$ -Polypeptids, das die Formel 137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146), 1-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146), oder 3-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146) aufweist und vom rekombinanten Plasmid pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) bzw. pGNB4 (FERM BP-549) codiert wird.
2. Rekombinantes Plasmid pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) oder pGNB4 (FERM BP-549).
3. Mikroorganismus transformiert mit einem rekombinanten Plasmid nach Anspruch 2, der Escherichia coli IGVT137 (FERM BP-547), Escherichia coli IGNC5 (FERM BP-550) oder Escherichia coli IGNB4 (FERM BP-549) ist.
4. Verfahren zur Herstellung eines Derivats eines menschlichen Interferon- $\gamma$ -Polypeptids nach Anspruch 1, wobei man einen Mikroorganismus nach Anspruch 3 in einem Medium züchtet und der Mikroorganismus das eingeschleuste DNA-Fragment exprimieren kann, das Polypeptid im Kulturmedium ansammelt und es daraus gewinnt.

Patentansprüche für folgenden Vertragsstaat: AT.

45

1. Rekombinantes Plasmid pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) oder pGNB4 (FERM BP-549).
2. Mikroorganismus transformiert mit einem rekombinanten Plasmid nach Anspruch 2, der Escherichia coli IGVT137 (FERM BP-547), Escherichia coli IGNC5 (FERM BP-550) oder Escherichia coli IGNB4 (FERM BP-549) ist.
3. Verfahren zur Herstellung eines Derivats eines menschlichen Interferon- $\gamma$ -Polypeptids, das die Formel 137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146), 1-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146) oder 3-Ser-137-Cys-IFN- $\gamma$  ( $\Delta$ 138-146) aufweist und vom rekombinanten Plasmid pGVT137 (FERM BP-547), pGNC5 (FERM BP-550) bzw. pGNB4 (FERM BP-549) codiert wird, wobei man in dem Verfahren einen Mikroorganismus nach Anspruch 2 in einem Medium züchtet und der Mikroorganismus das eingeschleuste DNA-Fragment exprimieren kann, das Polypeptid in dem Kulturmedium ansammelt und es daraus gewinnt.

55

FIG. 1

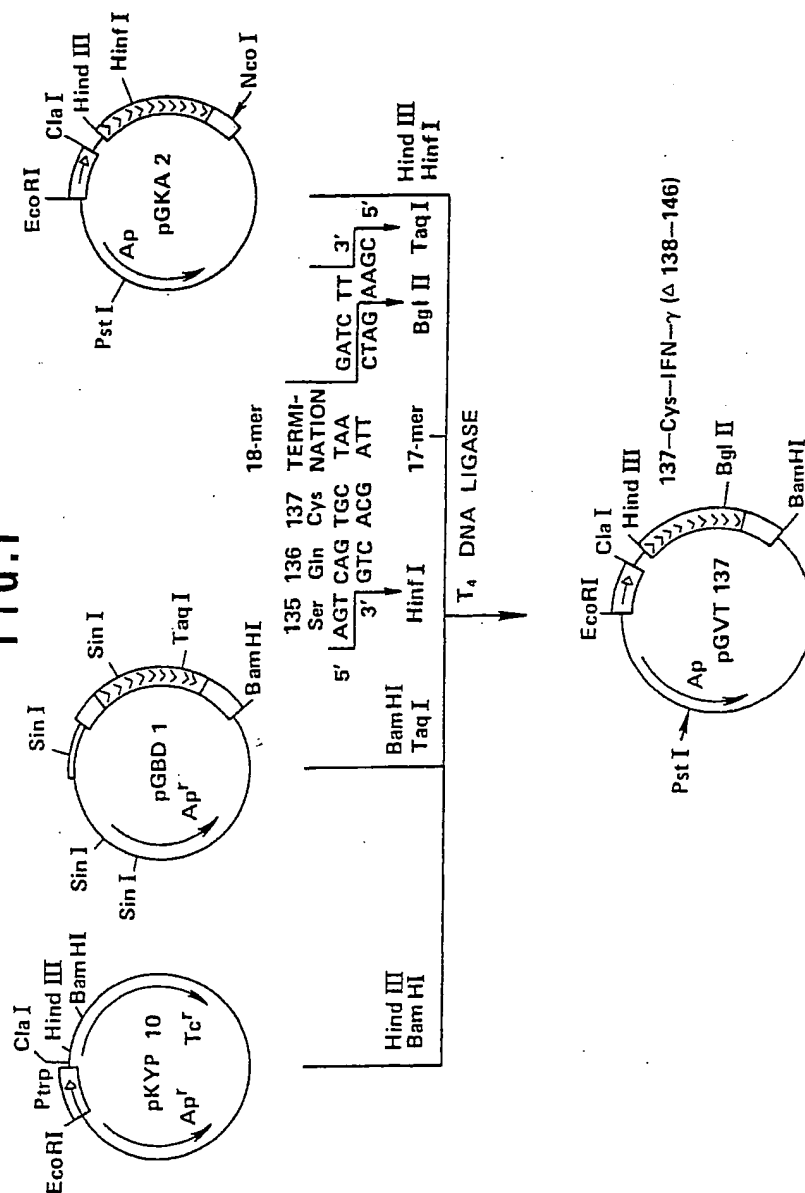


FIG. 2

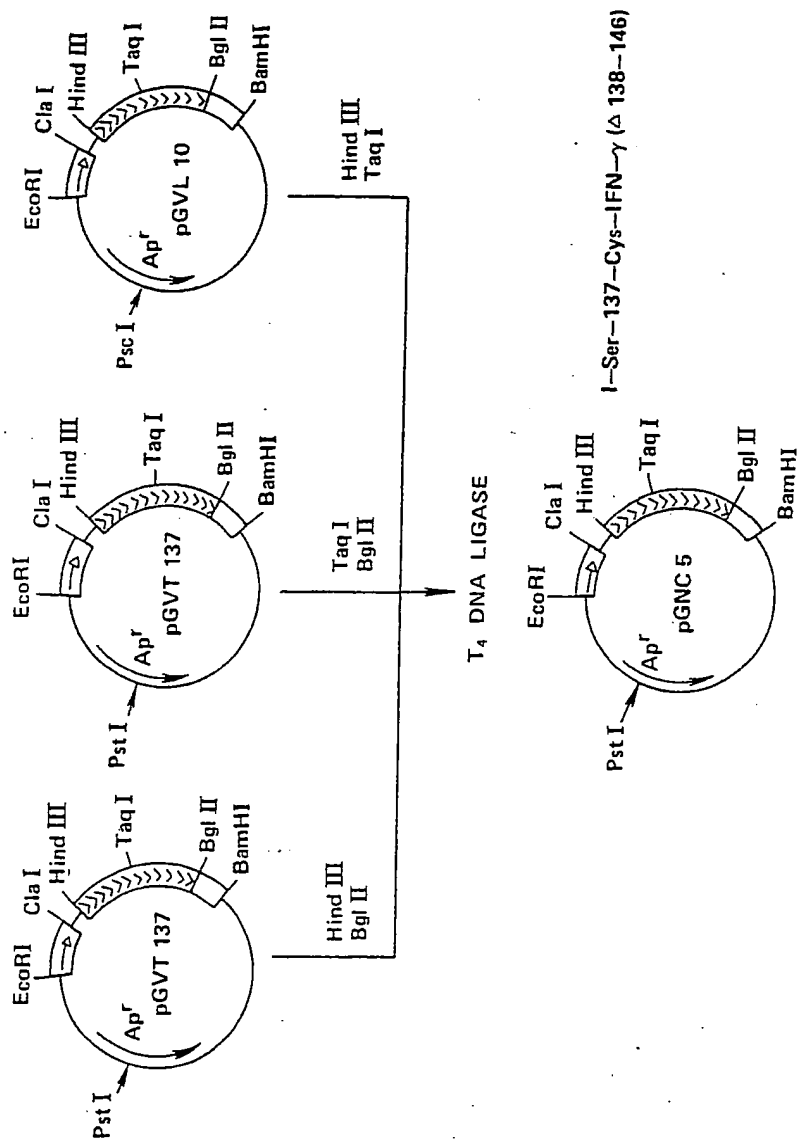


FIG. 3

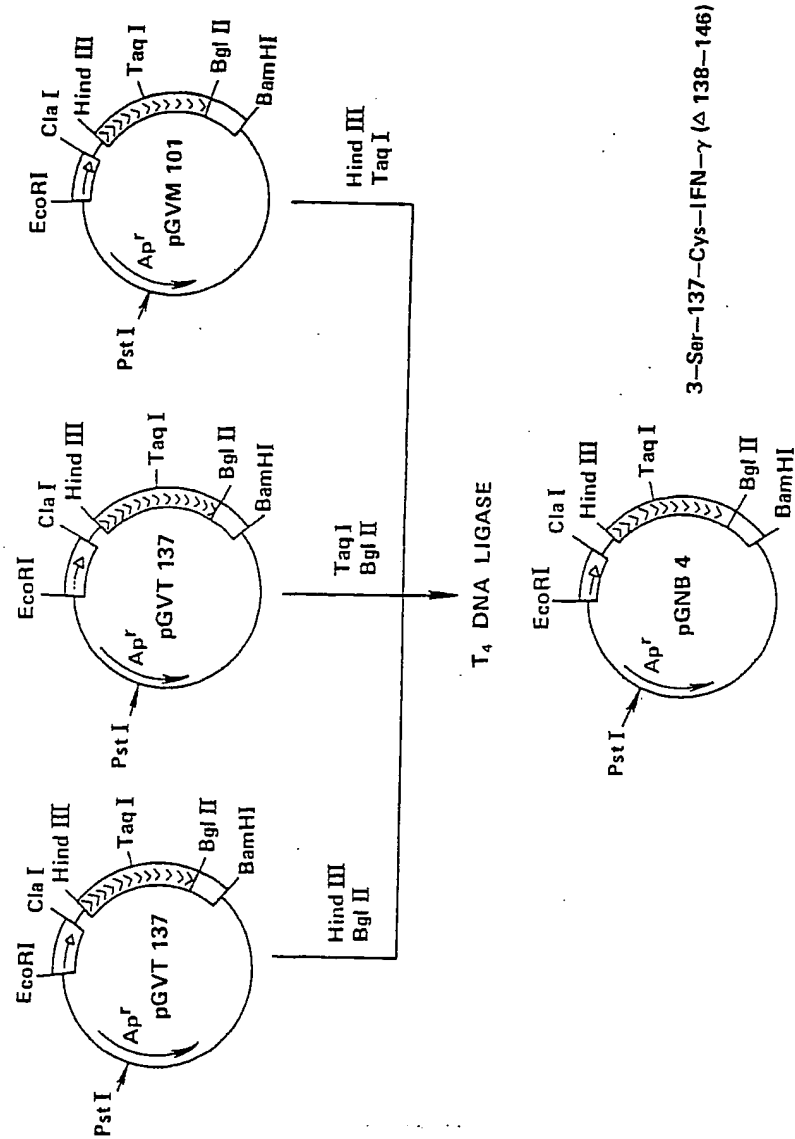
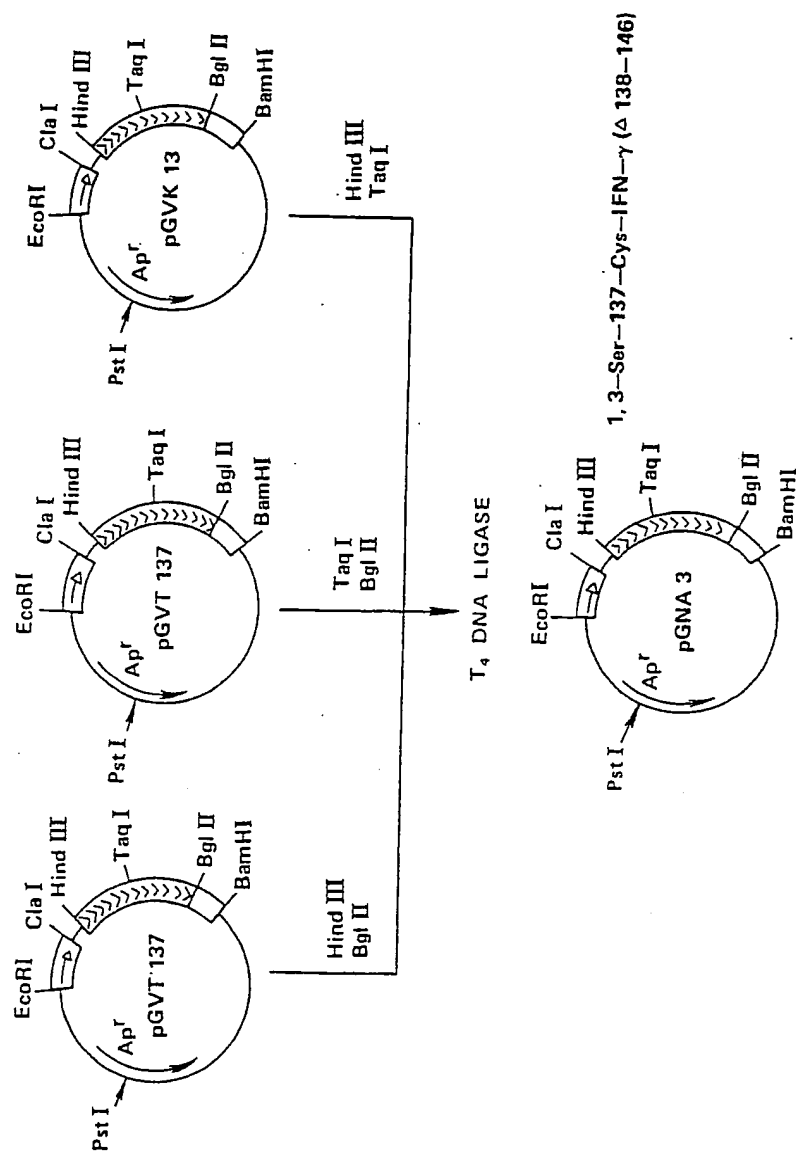


FIG. 4



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**